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(54) OLIGONUCLEOTIDE THERAPEUTIC AGENT AND METHODS OF MAKING SAME.

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Description

This invention relates to a therapeutic agent for selectiv ly blocking the translation of mRNA into a targeted protein by utilizing hybridization techniques of the type having messenger ribonucleic acid attached to oligonucleotides for in vivo protein synthesis regulation.

In the field of pharmacology, the use of therapeutic agents has long been recognized as an effective way to control diseases. Such agents are often used in treating bacterial or viral infection, chemical imbalances and the like, to cure, or at least mitigate, the diseased state. Although researchers occasionally discover new therapeutic agents after major breakthroughs have elucidated the molecular basis of a disease, more often they must rely on observing for antibiosis or modifying the chemical structures of functionally related chemicals.

With respect to antibiotic agents, some are quite effective at the outset, but over time many organisms become resistant or totally immune to their action. Additionally, very few effective antiviral agents have ever been developed, and without explicit, detailed knowledge of an infecting organism's physiology, the development of new operative agents remains haphazard.

In PNAS USA vol. 75, No. 1 (1978), pages 280 to 284 a particular tridecamer complementary to 13 nucleotides of the 3' and 5' reiterated terminal sequence of Rous Sarcoma virus 35 RNA is described. The tridecamer is observed to inhibit the virus production in chick embryo fibroblast tissue cultures. It is contemplated that the tridecamer might hybridize with the repetitive terminal sequence of the RNA and might interfere with the circularization step of the pro-virus DNA.

A corresponding tridecamer is described in PNAS USA vol. 75, No. 1 (1978), pages 285 to 288. The document refers to the ability of the tridecamer to prime virus RNA and to inhibit protein synthesis from the virion RNA. It is speculated that the inhibition might be due to hybridization of the tridecamer near potential initiation sites for viral protein synthesis.

In PNAS USA vol. 75, No. 3 (1978), pages 1271 to 1221 and PNAS USA vol. 74, No. 10, (1977), pages 4370 to 4374 in vitro methods of structural gene identification and mapping by DNA-mRNA hybrid-arrested cell-free translation are described. In the method DNA fragments of 500 to 1000 bases in length were hybridized with mRNA coding for known proteins at a temperature of 65°C for 20 min. or 48°C for 2 hr. It was found that translation of the hybridized regions of mRNA was reversably blocked at these conditions.

Angewandte Chemie International Edition, vol. 9 (1970), No. 9, pages 678 to 688 describes the use of modified nucleosides or nucleotides as antimetabolic agents for inhibiting the nucleic acid metabolism and particularly the synthesis of virus induced cDNA. The modified nucleosides or nucleotides are competing with their unmodified analogues for specific binding sites and positions within complex structures thereby blocking the physiological functions of the resulting products. It is further considered that the compounds may have immunological effect since DNA synthesis inhibitors are effective in suppressing the proliferation of sensitized lymphocytes and cell-mediated immunity. In this context reference is also made to oligonucleotides on the basis of the observation that antibody stimulation occurred with a gross mixture of DNA digest, the optimal size of the oligomers being indicated as tri- to hexanucleotides. It is speculated that the modified oligonucleotides would compete with their naturally occurring analogues for the protein binding sites, thereby blocking antibody production particularly in auto-immune diseases.

Finally, in Biochemistry, vol. 16, No. 9 (1977), pages 1988 to 1996 the preparation of a particular modified trinucleotide is described, which is complementary to a three base sequence common to the aminoacid accepting stem of most tRNAs. The document reports in vitro experiments, showing that the triplet is bound to tRNAs and inhibits amino acylation at a rate of 39% at 37°C. It was further shown that the triplet passes into the cells in vivo unspecificially reducing peptide synthesis for an interval of about 24 hours.

No therapeutic agent has up to now been described in the prior art, by means of which it would be possible to selectively block the translation of mRNA into a particular, targeted protein without effecting the synthesis of other proteins.

Thus, there exists a definite need for a therapeutic agent that is versatile and inexpensive and yet both extremely specific and effective. The present invention fulfils these needs.

SUMMARY OF THE INVENTION

The present invention provides a methodology of identifying and constructing therapeutic agents for use in living organisms that substantially reduces the uncertainty surrounding the development of new antagonists, significantly increasing the scope of materia medica. Moreover, the agent construction of the present invention lends itself readily to simple manufacture, even in large quantities, is extremely effective in use, and attains its improved results without undue cross-reactions.

According to the invention a stabilized oligonucleotide, preferably in a phosphotriester

form, is provided having a base sequence substantially complementary to a portion of messenger ribonucleic acid coding for a biological component of an organism. Due to the complementary nature of the oligonucleotide and the messenger ribonucleic acid, the two components can readily hybridize under appropriate conditions to control synthesis of the organism's biological component and, if the protein is vital to the organism's viability, to act as an antibiotic.

A method, in accordance with the present invention, of developing therapeutic agents may typically include the steps of: providing a base sequence of an organism's nucleic acid that contains at least a portion of the genetic information for a biological component of the organism, and synthesizing an oligonucleotide the sequence of which is derived from the base sequence, for subsequent hybridization with the messenger ribonucleic acid specific for the biological component. The biological component may be a vital protein, or simply a hormone such as the gonadotropin, follicle stimulating hormone. The order of the base sequence may be determined from deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), preferably messenger ribonucleic acid (mRNA). Alternatively, the desired oligonucleotide base sequence may be determined from the biological component's sequence, as when the biological component is a protein. The oligonucleotide of the invention has fourteen to twenty-three bases, and for increased stability, is transformed to a more stable form, such as a phosphotriester form, to inhibit degradation during use.

To produce large quantities of the oligonucleotide, it may be synthesized chemically, such as in automated machines, or inserted into a plasmid, such as pBR322, for cloning. The plasmid insertion may be accomplished with linker base sequences, such as GATTCGAATC or CTAAGCTTAG, which are susceptible to degradation by Hind III restrictive nuclease or Alu I restriction nuclease. When the order of the base sequence has not been determined, the base sequence can be cloned and then cross-hybridized against messenger ribonucleic acid from the other sources to remove base sequences non-specific to the target.

Another aspect of the present invention is a method of selectively controlling activity of one or more biological components in a cell without substantially interfering with the activity of other biological components in the cell. The method includes the steps of forming an oligonucleotide having a base sequence substantially complementary to a portion of mRNA coding for the specific biological component, and introducing the oligonucleotide into the cell for hybridization with the selected mRNA. This causes blocking of the translation of the mRNA into protein. The oligonucleotide has fourteen to twenty-three bases. The target mRNA can code for a protein, such as the hormone, follicle stimulating hormone. This hormone has an alpha and beta chain, and the oligonucleotide should be specific for the mRNA coding for the beta chain to avoid cross-reacting with other gonadatropin mRNA. A suitable oligonucleotide base sequence would be AC-CACGCGR$_1$CCR$_2$ATGACGATGTG, wherein R$_1$ is G or T and R$_2$ is also G or T.

In accordance with another aspect of the present invention, a method is provided for inhibiting the infection of a host organism by a foreign organism. This method entails isolating a base sequence containing at least a portion of the genetic information coding for a vital protein from the foreign organism's nucleic acid; synthesizing an oligonucleotide, the order of which is derived from the base sequence and substantially complementary to the messenger ribonucleic acid coding for the protein; and treating the foreign organism with an effective amount of the oligonucleotide to hybridize with a portion of the messenger ribonucleic acid and block translation of the protein. The oligonucleotide, which can be a deoxyribonucleotide, is transformed to a more stable form, such as a phosphotriester form, to inhibit degradation, and the order of the sequence determined prior to its synthesis. Further, to increase the oligonucleotide's specificity, it may be cross-hybridized against mRNA from different organisms, such as the host organism, to remove non-unique oligonucleotide sequences.

It will be appreciated from the foregoing that the present invention satisfies a long existing need for improved methods of developing therapeutic agents for use in living organisms, and represents a significant advance over previously available methods, principally because it is very versatile, and yet provides a very specific agent against a biological component. Other aspects and advantages of the invention will become apparent from the following more detailed description taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a flow diagram showing the central dogma of molecular biology;

FIG. 2 is a flow diagram showing normal translation of messenger ribonucleic acid (mRNA) into T protein, as well as a synthetic oligonucleotide of the present invention blocking translation of the T protein;

FIG.3 is a list of the viral deoxyribonucleic acid (DNA) code specific for SV-40 T protein, and the related mRNA and oligonucleotide;

FIG.4 is a flow diagram showing the construction of high yield plasmids containing the T protein oligonucleotide;

FIG.5 is a flow diagram showing the use of restriction nucleases to cleave the plasmids to give a purified T protein oligonucleotide;

FIG. 6 is a flow diagram showing the treatment of a DNA sequence to form DNA polyphosphotriesters; and

FIG. 7 is a chart showing the partial amino acid sequence of follicle stimulating hormone, as well as the predicted mRNA sequence and related oligonucleotide family.

DETAILED DESCRIPTION OF THE INVENTION

Referring now to the drawings, and particularly to FIGS. 1 and 2, there is shown the so-called "central dogma" of the molecular biology of life. Basically, it is now accepted that deoxyribonucleic acid (DNA) carries the genetic code for almost all living organisms. The code exists in the form of an organized sequence of four nucleotide bases attached to a phosphorylated, deoxyribose sugar backbone. Generally, DNA exists in the form of a double strand helix formed of two oppositely directed strands, which are held together in an opposing manner by various weak forces.

A primary constituent of these weak forces are the so-called hydrogen bonds that exist between nuclectides on the opposing strands. The four bases, adenine (A), cytosine (C), guanine (G), and thymine (T), form hydrogen bonds generally in only one fashion: A with T and C with G. Thus, by knowing the sequence of one strand, the sequence of the second strand can be readily determined.

Another aspect of the central dogma is that proteins are produced indirectly from the DNA strand, through messenger ribonucleic acid (mRNA). Apparently, mRNA, which has the same structure as single stranded DNA except with a ribose backbone and with uracil (U) replacing thymine, is transcribed directly from one DNA strand and has an essentially opposite base sequence, i.e., if a DNA strand sequence is 5 ...ACGT...3' the transcribed mRNA sequence is 3'...UGCA...5'.

An additional aspect of the central dogma relates to the translation of mRNA into proteins. Briefly, excluding initiation sites and the like, each three nucleotide base grouping (triplet code) codes for one amino acid of a protein. Therefore, by knowing the mRNA sequence of a protein, its amino acid sequence can generally be determined. However, the reverse is not true, that is, knowing the amino acid sequence does not guarantee precise knowledge of the mRNA sequence. This stems from the fact that there exist 64 (4^3) possible triplet codes, yet there exists only about twenty amino acids, allowing some amino acids to have multiple triplet codes.

The similarity in the structure of DNA and mRNA strands creates interesting effects. Most notably, if complementary DNA and RNA strands exist contemporaneously in a solution, under certain established conditions the strands can anneal, forming hybrids. One important factor in proper annealing is the melting temperature, which can be calculated according to Britten et al, Methods of Enzymology 29:363 (1974).

In accordance with the present invention, a synthetic oligonucleotide having a base sequence of 14 to 23 bases capable of substantially matching that of a chosen mRNA is provided for hydridization with that mRNA. Once such a hybrid exists, the translation of the mRNA into protein becomes significantly inhibited. If the inhibited protein is vital to an organism's survival, the organism's viability, i.e., either growth or continued life, is jeopardized. Importantly, the oligonucleotide can be designed specifically for the mRNA coding for just one protein, and should not cross-react with mRNA for other proteins.

The method of developing the oligonucleotide entails basically two steps. As described more fully below, a possible first step is to determine the appropriate sequence of the mRNA specific for the protein to be inhibited, and a second step is to manufacture an oligonucleotide complementary to the mRNA. Once made, the oligonucleotide is treated into e.g. a phosphotriester form for increased stability.

A variety of techniques exists for determining nucleic acid base sequences. In many instances the sequence of the mRNA or the gene have been determined and published in the biochemical literature. In fact, researchers have determined the complete nucleotide sequence for the SV-40 virus (Reddy et al, Science 200:494 (1978)). As is well known, an alternative method entails isolating and purifying mRNA in sufficient quantities to permit sequencing studies, but this can prove difficult due to the relative instability and, in some cases, extreme rarity of many mRNA sequences.

Still another method for determining nucleic acid base sequences requires resolving the amino acid sequence from the target protein. After determining the amino acid sequence of the target protein in purified form, a sequential degradation utilizing commercially available protein sequences (e.g. from Beckman Instruments, Fullerton, California) can be used to provide the amino acid sequence. Once this has been obtained, knowledge of the triplet code can be applied to give prospective base sequences. An example of such a process for the hormone glucagon can be found in Tullis et al,

Biochemical and Biophysical Research Communications 93:941 (1980).

Once the sequence of the appropriate nucleic acid and the desired mRNA sequence hav been determined, an oligonucleotide, such as deoxyribonucleotide, complementary to the mRNA can be constructed. A number of synthetic techniques are known, most typical is the diester approach described by Agarwal et al, Nature 227: 27 (1970), and oligonucleotide synthesizers may be purchased commercially from Vega Biochemicals, P.O. Box 11648, Tucson, Arizona and Biologicals, Inc., Toronto, Canada.

If the desired oligonucleotide sequence is unknown, a suitable oligonucleotide can be prepared as follows. After isolating mRNA from a target organism, multiple copies are made, preferably in the form of DNA, so-called copy DNA (cDNA). This cDNA is then cross-hybridized against mRNA isolated from other organisms, and any cDNA hybridizing is removed. The remaining cDNA is specific only to the target organism, and can serve as the therapeutic agent.

In order to obtain a high degree of specificity, an oligomer of 14 to 23 bases is constructed. Although shorter sequences would work, these sequences provide higher specificity. This can readily be seen mathematically. Whereas a ten unit polymer chosen from four bases can have 4^{10} - (1,048,576) random combinations, a 20-unit polymer has 4^{20} random combinations, which equals 1.09×10^{12} (1,090,000,000, 000).

In spite of the added difficulty in making oligonucleotides of twenty units in comparison to ten bases, it is warranted because the exponential increase in complexity reduces undesirable cross-reactivity. It has been estimated that a mammalian cell contains about 2×10^8 nucleotides of RNA complexity or, in other words, approximately 200 million nucleotides of unique sequence mRNA, which is equivalent to about 30,000 mRNA sequences. The probability that one of those sequences contains a randomly chosen 20-unit polymer is approximately one in fifty-five hundred. In comparison, a ten-unit polymer has about a one hundred and ninety to on chance for random cross-reaction.

The present invention is illustrated by, but not limited to, the following examples.

EXAMPLE I

SV-40 virus manufactures a vital protein commonly known as the "T protein" or "T antigen protein". As noted earlier, the complete genetic code for the SV-40 virus has been determined, and it is known that residues 5091 to 5071 on the viral genome code for a portion of the T protein mRNA. The sequence of these residues, the viral T protein mRNA sequence, and the designed T protein specific oligonucleotide are shown in Fig. 3. In this case, the T protein specific oligonucleotide is complementary to th viral T protein mRNA, and identical to the portion of the viral DNA code.

Prior to testing the effectiveness of the T protein specific oligonucleotide in vivo, the oligonucleotide can be mixed with total mRNA from an organism to check for cross-reactivity. If it hybridizes, then a different portion of the viral genome coding for the T protein should be utilized. Otherwise, the oligonucleotide is ready for further testing.

Further testing requires growth of S-40 virus. For purposes of these experiments, SV-40 virus is grown and titered in African green monkey cells, such as the cell line BSC-1, according to Hopps et al, Journal of Immunology 91:416 (1963). The identity of the virus can be confirmed by the following methods:

a) checking for tumor production after innoculation of the newborn hamsters with the virus;

b) neutralization of the virus by anti-SV-40 antiserum; and

c) reaction of the infected cells with anti-SV-40 T antigen directed antibodies prepared by standard techniques.

The isolation of SV-40 mRNA can be accomplished as follows. Total RNA is first obtained by the guanidine hydrochloric acid extraction procedure using glassware previously baked and treated with diethylpyrocarbonate to remove traces of RNase as taught by Cox et al, Methods in Enzymology 12B:120 (1968). The A+ RNA is isolated on oligo-dTcellulose, which can be obtained from Collaborative Research, Waltham, Massachusetts, or P. L. Biochemicals, Inc., Milwaukee, Wisconsin, utilizing the technique described by Bantle et al, Analytical Biochemistry 72:413 (1976). The RNA fractions are assayed for purity and intactness by electropheresis according to the method described in Bailey et al, Analytical Biochemistry 70:75 (1976). Also, the RNA can be assayed for translatability in the wheat embryo in vitro system described in Marcus, et al, Methods in Enzymology: 30:749 (1974). The in vitro translation products are monitored on sodium lauryl sulphate 9% polyacrylamide gels as described in Laemmeli, Nature 227:680 (1970).

This purified A+ mRNA containing the viral mRNA sequences can hybridize to the synthetic oligonucleotide at 37°C in 0.5 M sodium phosphate buffer, pH 6.8, containing 0.2% sodium lauryl sulphate. Solutions containing about 1 mg A+ mRNA and synthetic oligonucleotide at a concentration of about 100 ug/ml are heated to 100°C for 1-2 minutes, then cooled to 37°C and allowed to anneal. The extent of the hybridization reaction as a func-

tion of time may be monitored on a gel filtration column.

Actually, while any theoretically suitable temperature may be used for the hybrid formation, temperatures ranging from 0°C to about 80°C provide for good hybridization, but preferred temperatures range from about 10°C to about 40°C. Generally, the optimal annealing temperature for the formation of specific hybrids is believed to be about 20°C to 25°C below their melting temperature. Synthetic oligonucleotides operating at 37.5°C should thus be designed on the basis of their base sequence and length, such that the melting temperature is between about 57°C and 62°C when tested under approximately physiological conditions.

For hybridization testing the ratio of the synthetic oligonucleotide to its mRNA complement is generally about 30:1. Lower ratios are acceptable, however, sequences below about 3:1 can cause lower hybrid formation. Control reactions utilizing yeast RNA or globin mRNA can be used, and should show no detectable hybrids, indicating hybridization specificity only to SV-40 mRNA. Also, thermal denaturation profile studies and comparison of the kinetics of hybridization can confirm that the synthetic oligonucleotide reacts only with SV-40 mRNA sequences.

Once it is shown that the oligonucleotide hybridizes to the isolated SV-40 mRNA, in vitro translation tests can be attempted utilizing the wheat embryo system (described previously) to show that the hybrid is not translated. Basically, upon introduction of SV-40 mRNA into the wheat embryo system, the system produces large T antigen protein. However, when an equal amount or more of synthetic oligonucleotide is also added to the system, T antigen protein synthesis can be substantially inhibited, without interference with synthesis of other SV-40 proteins whose mRNA was also introduced.

Testing of the oligonucleotide in vivo can be accomplished-by adding the oligonucleotide to cultures of cells infected with SV-40. Synthesis of T antigen protein should be inhibited significantly in about six hours, and SV-40 growth should be strongly inhibited within about 24 hours. The growth of control cultures should be largely unaffected.

The synthetic oligonucleotide of the present invention may be mass produced according to common cloning techniques, such as those developed in the art to clone the gene for proinsulin. Alternatively, the oligonucleotide can be chemically synthesized in commercially available equipment (described previously). Briefly, the cloning method entails enzymatic insertion of the oligonucleotide into a bacterial gene carried on a larger piece of DNA, known as a plasmid. The plasmid can be incorporated into a suitable host bacteria, and multiple copies made as the bacteria multiply as in Boyer and Cohen, U. S. Patent No. 4,237,224.

More particularly, and with reference to FIGS. 4 and 5, the cloning plasmid designated as pBR322, available from Bethesda Research Labs, Inc., Rockville, Maryland, can be used to mass produce the T protein specific oligonucleotide. Using standard techniques, the oligonucleotide is converted to double stranded form and then a terminal $5'PO_4$ is added to each of the 5' termini with polynucleotide kinase to permit subsequent joining through T-4 ligase. The reaction conditions for forming the 5' termini can be found in Richardson, Progress in Nucleic Acids Research 2:815 (1972).

After purification of the double stranded oligonucleotide by chromatography on hydroxylapatite columns, it is inserted into the plasmid. Because the oligonucleotide is blunt ended, the plasmid should not have uneven or "sticky" ends. To remove sticky ends from the plasmid, S1 nuclease or other single strand specific nucleases can be utilized. A general description of methods for using restriction nucleases can be found in Nathans and Smith, Annual Review of Biochemistry 44: 273 (1975).

For best results, a linker system between the oligonucleotide and the plasmid can be utilized, specifically a linker having both Hind III and Alu I enzymatic cleaving sites. As seen in FIG. 4, one such linker has a sequence: 5'... CTAAGCTTAG...3'. This sequence represents a double stranded, bisymmetric molecule containing a recognition sequence both for Alu I (AGCT) and for Hind III (AAGCTT). Utilizing DNA ligase under standard conditions, this molecule can be ligated to the oligonucleotide to form linker-oligonucleotide-linker molecules. Similarly, the linked oligonucleotide can be introduced into linearized blunt-ended, Hind III cleaved pBR322 carrier molecules.

After ligation, the plasmid has resumed its covalently closed circular configuration with the linker-oligonucleotide incorporated, all of which is shown in FIG. 5 as pT-protein oligonucleotide. The recircularized plasmid is then used to transform a suitable bacterial host such as E. coli. The methods for transformation and selection of transformants are known in the art and described in detail in Cohen and Boyer, U.S. Patent No. 4,237,224.

Once the transformed bacteria containing the ligated plasmid p-oligonucleotide have been grown to high density and produced large amounts of the ligated plasmid, the oligonucleotide is ready for purification. After the plasmid has been removed from the mature cells, the plasmid is treated with appropriate restriction endonucleases. As illustrated in FIG. 5, the plasmid is first cleaved with Hind III

to give various by-products, including linker-T-protein-oligonucleotide-linker and fragments of the original plasmid. These are readily separated utilizing gel electrophoresis or high pr ssure liquid chromatography. Further cleavage of the isolated linker-oligonucleotide-linker with the endonuclease Alu I yields pure double stranded oligonucleotide and partially degraded linker. These can also be separated based on their size differences.

As shown in FIG. 6, the oligonucleotide can then be modified to a nuclease resistant phosphotriester form utilizing the reaction described in Miller et al, Biochemistry 16:1988 (1977). Basically, the oligonucleotide is first acylated using 50% acetic anhydride-pyridine during an overnight incubation period. The product is precipitated and isolated from ether. The phosphotriester can then be formed utilizing 30% ethanol in anydrous 2, 6 lutidine (30%), NN-dimethyl formamide (30%) and p-toluene sulfonyl chloride (17%), and reacting for about 6 hours. The protecting acetyl groups are then hydrolyzed by the addition of 0.5 volumes of concentrated ammonium hydroxide, followed by incubation for about 1 hour at 55°C, The final oligonucleotide product in the ethyl phosphotriester form can then be isolated on paper chromatography or high pressure liquid chromatography.

It is believed that transforming the oligonucleotide to a phosphotriester form will improve the oligonucleotide's stability in vivo due to an enhanced resistance against various degradative enzymes. However, the oligonucleotide will eventually degrade because of spontaneous de-ethylation, which leaves the molecule unprotected. Indeed, by controlling the initial level of ethylation, the in vivo degradation rate can be controlled. A further advantage of a phosphotriester form is believed to be an increase in the oligonucleotide's ability to penetrate a cell membrane.

EXAMPLE 2

A synthetic oligonucleotide capable of inhibiting the synthesis of follicle stimulating hormone (FSH), a protein hormone produced by the pituitary that functions in the maturation of ova in females and sperm cells in males, can also be constructed. It is known that FSH is composed of two chains, alpha and beta, the amino acid sequence of which has been determined for several animal species. Interestingly, the alpha chain of FSH is common to other gonadotropic hormones, including thyroid stimulating hormone, luteinizing hormone, and chorionic gonadotropin, while the beta chain varies. Therefore, to selectively shut off the synthesis of FSH without substantially affecting the other gonadotropins, the oligonucleotide must be specific for the mRNA coding for the beta chain.

The sequence of the beta chain amino acids 32 through 40 is shown in FIG. 7. As discussed earlier, it is possible to predict the mRNA base sequence for these amino acids, although not with absolute certainty. The points of uncertainty are indicated by the letter "X" in the predicted mRNA sequence. Thus, the resultant oligonucleotide family consists of eight possible 26 base sequences; the potential alternate bases are shown in parentheses below the primary base sequence.

By beginning with the projected mRNA sequence for the 33rd through 40th amino acids, it can be seen that four different 23 base oligonucleotides exist that could correspond to the FSH mRNA. The sequences could be as follows, reading from the 5' end: GTGTAGCAGTAGCCGGCGCACCA, GTGTAGCAGTATCCGGCGCACCA, GTGTAGCAGTAGCCTGCGCACCA, and GTGTAGCAGTATCCTGCGCACCA.

One of these four sequences should be precisely correct and thus able to hybridize fully with the FSH mRNA. To determine the best sequence, a hybridization test against FSH mRNA, with subsequent purification on hydroxylapatite or other suitable column, can be performed as previously described. Once the best sequence has been determined, it is placed in a plasmid or chemically synthesized, as described above, for bulk synthesis. This oligonucleotide should substantially inhibit the synthesis of FSH in vivo.

From the foregoing, it will be appreciated that the present invention provides a systematic method of designing new therapeutic agents for use in living organisms and that this method is versatile and inexpensive. Further, the oligonucleotide produced in accordance with the present invention is extremely effective and specific, enabling selective control of protein synthesis in a living organism.

While several particular forms of the invention have been illustrated and described, it will be apparent that various modifications can be made without departing from the spirit and scope of the invention. Accordingly, it is not intended that the invention be limited, except as by the appended claims.

Claims

Claims for the following Contracting States : BE, CH, DE, FR, GB, LI, NL, SE

1. Therapeutic agent for selectively blocking the translation of mRNA into a targeted protein, comprising a stabilized oligonucleotide of 14 to 23 bases having a base sequence substantially complementary to a portion of the coding region of the mRNA coding for said targeted protein.

2. Therapeutic agent according to claim 1, characterized in that the oligonucleotide is in a phosphotriester form.

Claims for the following Contracting State : AT

1. A method for preparing a therapeutic agent for selectively blocking a translation of mRNA into a targeted protein comprising the manufacturing of a stabilized oligonucleotide of 14 to 23 bases having a base sequence substantial complementary to a portion of the coding region of the mRNA coding for said targeted protein and formulating the same into a therapeutic agent.

2. A method for preparing a therapeutic agent according to claim 1, characterized in that the oligonucleotide is prepared in a phosphotriester form.

Revendications
Revendications pour les Etats contractants suivants : BE, CH, DE, FR, GB, LI, NL, SE

1. Agent thérapeutique qui bloque d'une manière sélective la traduction de l'ARNm d'une protéine cible, comportant un oligonucléotide stabilisé de 14 à 23 bases dont une séquence de bases est en grande partie complémentaire d'une fraction de la région codante de l'ARNm codant pour ladite protéine-cible.

2. Agent thérapeutique selon la revendication 1, caractérisé en ce que l'oligonucléotide est sous forme de phosphotriester.

Revendications pour l'Etat contractant suivant : AT

1. Procédé de fabrication d'un agent thérapeutique qui bloque d'une manière sélective une translation d'ARNm d'une protéine cible, comprenant la préparation d'un oligonucléotide stabilisé de 14 à 23 bases dont une séquence de bases est en grande partie complémentaire d'une fraction de la région codante de l'ARNm codant pour ladite protéin cible et la formulation du même en un agent thérapeutique.

2. Procédé de fabrication d'un agent thérapeutique selon la revendication 1, caractérisé en ce que l'oligonucléotide est préparé sous forme de phosphotriester.

Patentansprüche
Patentansprüch für folg nde Vertragsstaaten : BE, CH, DE, FR, GB, LI, NL, SE

1. Therapeutisches Agens zum selektiven Blokkieren der Translation von mRNA in ein Zielprotein, welches ein stabilisiertes Oligonukleotid mit 14 bis 23 Basen umfaßt, das eine Basensequenz aufweist, die im wesentlichen komplementär zu einem Bereich der kodierenden Region der mRNA ist, welche für das Zielprotein kodiert.

2. Therapeutisches Agens nach Anspruch 1, **dadurch gekennzeichnet**, daß das Oligonukleotid in Form eines Phosphotriesters vorliegt.

Patentansprüche für folgenden Vertragsstaaten : AT

1. Verfahren zum Herstellen eines therapeutischen Agens zum selektiven Blockieren einer Translation von mRNA in ein Zielprotein, welches das Herstellen eines stabilisierten Oligonukleotids mit 14 bis 23 Basen mit einer Basensequenz, die im wesentlichen komplementär zu einem Bereich der kodierenden Region der mRNA ist, die für das Zielprotein kodiert, und das Formulieren desselben zu einem therapeutischen Agens umfaßt.

2. Verfahren zum Herstellen eines therapeutischen Agens nach Anspruch 1, **dadurch gekennzeichnet**, daß das Oligonukleotid in Form eines Phosphotriesters hergestellt wird.

FIG.1

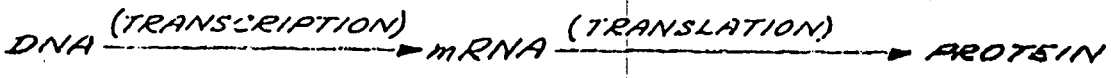

FIG.2

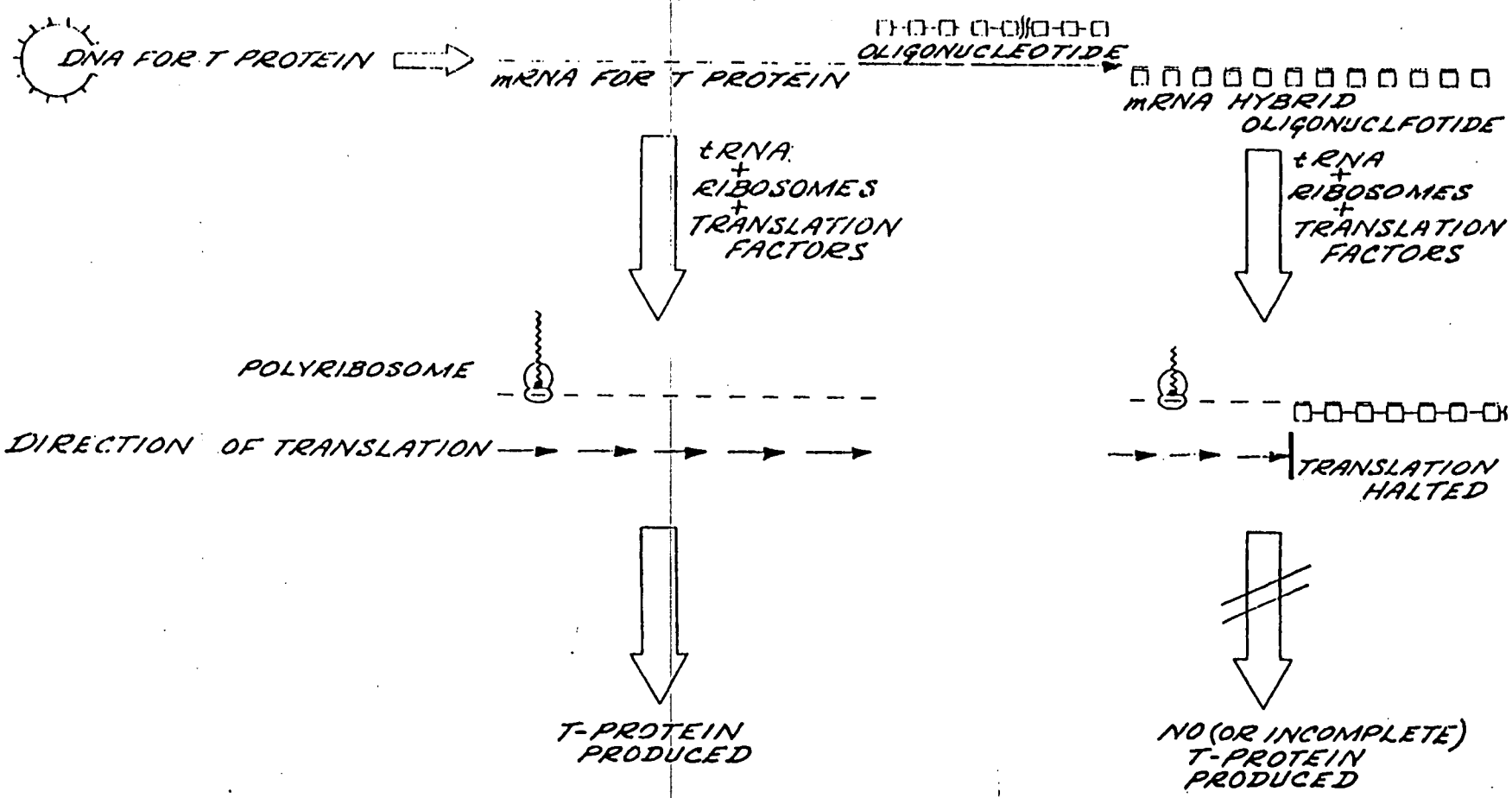

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FIG. 3

OLIGONUCLEOTIDE SPECIFIC FOR SV-40 T PROTEIN

VIRAL DNA (CODING) 3'...GAAACGTTTCTACCTATTTC...5'

VIRAL T PROTEIN mRNA 5'...CUUUGCAAAGAUGGAUAAAG...3'

TPROTEIN SPECIFIC OLIGONUCLEOTIDE 3'...GAAACGTTTCTACCTATTTC...5'

FIG. 7

OLIGONUCLEOTIDE SPECIFIC FOR FOLLICLE STIMULATING HORMONE

PROTEIN SEQUENCE:
N TERMINUS....THR TRP CYS ALA GLY TYR CYS TYR THR...C TERMINUS

mRNA PREDICTED:
5'END ACX UGG UGC GCX GGX UAC UGU UAC ACX 3'END

FSH OLIGONUCLEOTIDE FAMILY:
3'END TCG(T) ACC ACC CGG(T) CCG(T) ATG ACG ATG TG- 5'END

FIG.4

CONSTRUCTION OF HIGH YIELD PLASMID CONTAINING THE T PROTEIN OLIGONUCLEOTIDE

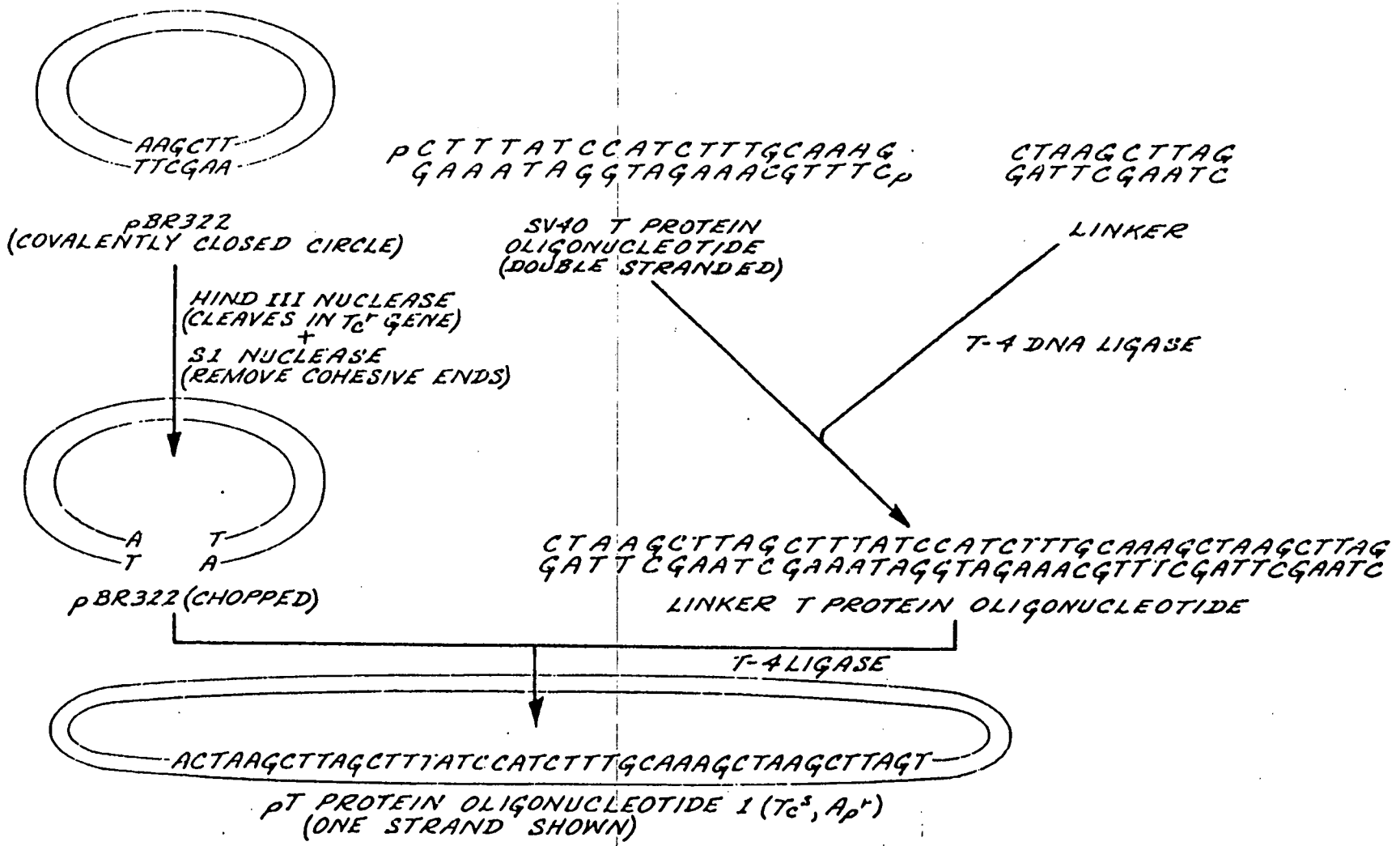

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FIG. 5

PRODUCTION OF SV-40 T PROTEIN OLIGONUCLEOTIDE FROM pT OLIGONUCLEOTIDE USING RESTRICTION NUCLEASE CLEAVAGE TO RELEASE FRAGMENTS.

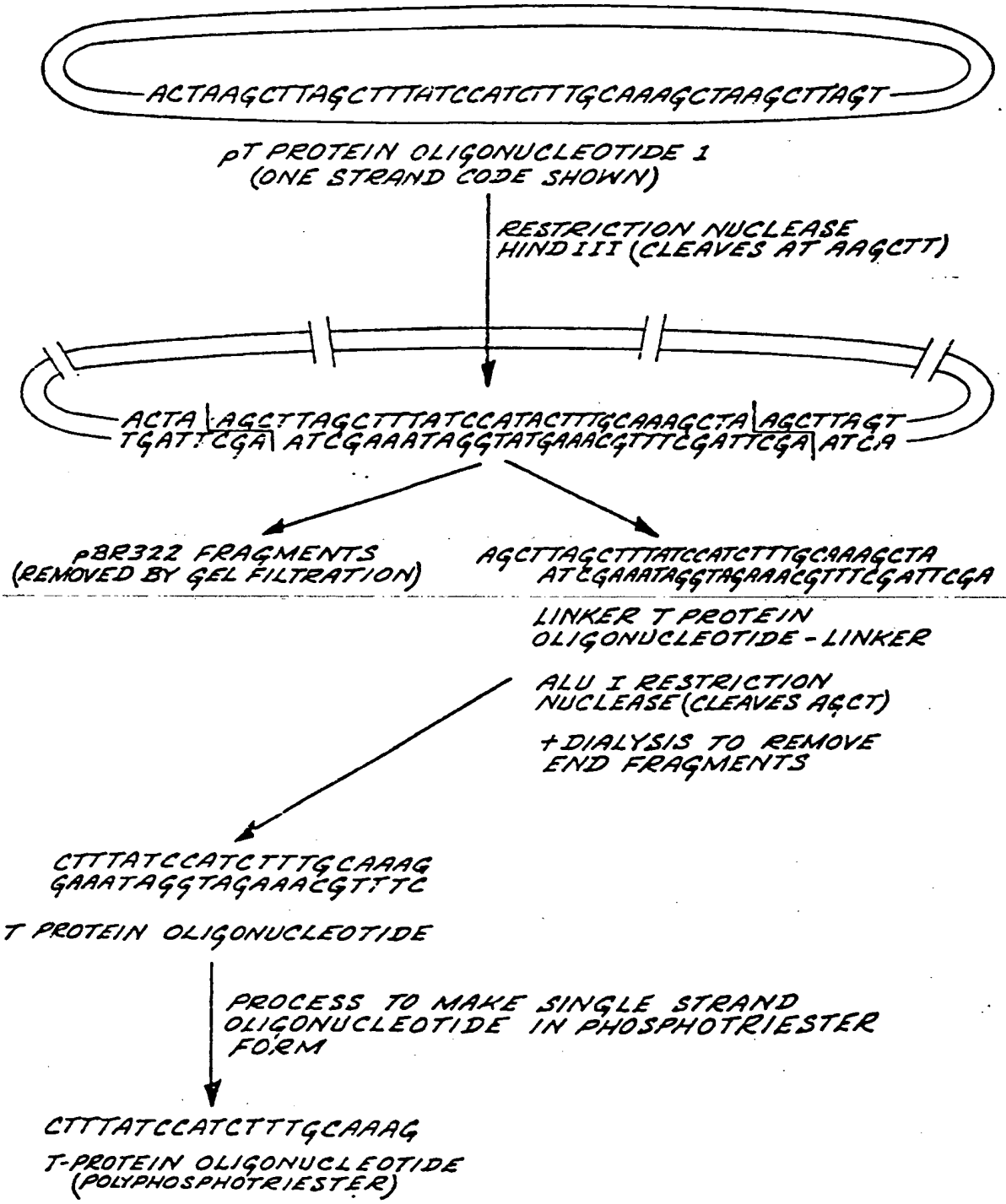
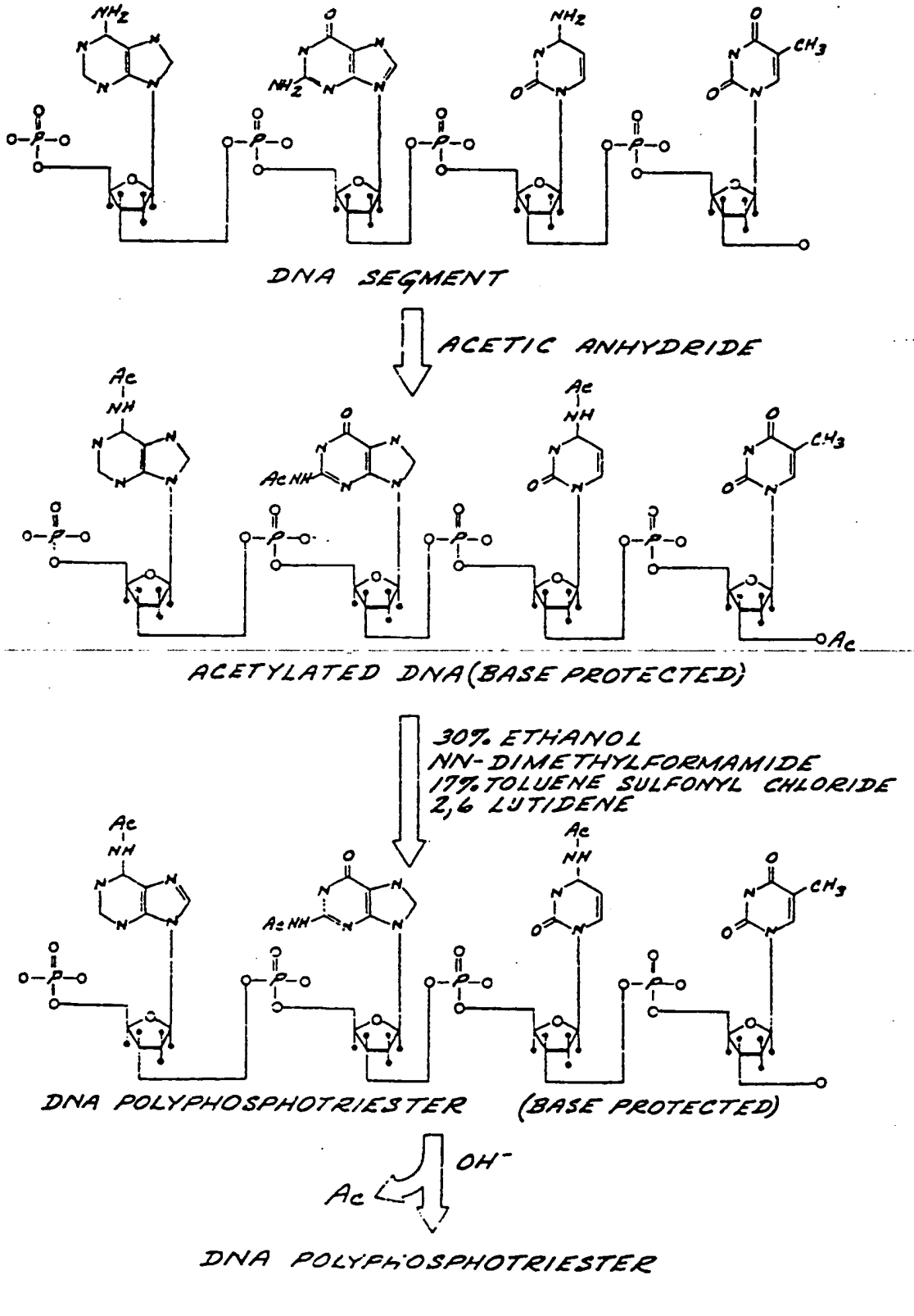


FIG.6


DNA SEGMENT
ACETIC ANHYDRIDE
ACETYLATED DNA (BASE PROTECTED)
30% ETHANOL
NN-DIMETHYLFORMAMIDE
17% TOLUENE SULFONYL CHLORIDE
2,6 LUTIDENE
DNA POLYPHOSPHOTRIESTER (BASE PROTECTED)
OH⁻
DNA POLYPHOSPHOTRIESTER

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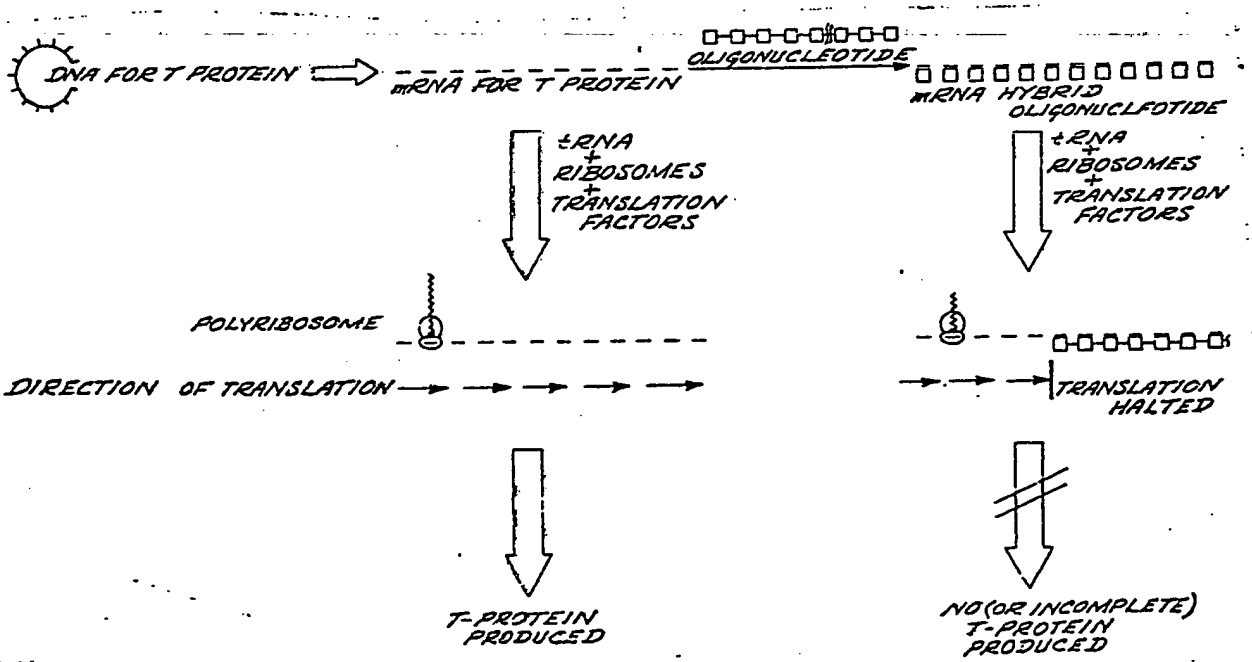

(57) Abstract

For use in controlling biologic functions in an organism, a stabilized oligonucleotide, preferably in a phosphotriester form, having a base sequence substantially complementary to a portion of messenger ribonucleic acid coding for a biological component, such as a protein, of the organism. The oligonucleotide has about f urteen bases or more, such as twenty-three bases, and can be a deoxyrib nucleotide. The oligonucleotide sequence can be derived from the organism's ribonucleic or deoxyribonucleic acid that codes for a vital protein, and can be synthesized in bulk either chemically or by insertion into a plasmid.

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OLIGONUCLEOTIDE THERAPEUTIC AGENT AND METHODS OF MAKING SAME

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BACKGROUND OF THE INVENTION

This invention relates generally to controlling biologic functions, such as for antibiotic purposes, and more particularly, to utilizing hybridization techniques of the type having messenger ribonucleic acid attached to oligonucleotides for in vivo protein synthesis regulation.

In the field of pharmacology, the use of therapeutic agents has long been recognized as an effective way to control diseases. Such agents are often used in treating bacterial or viral infections, chemical imbalances and the like, to cure, or at least mitigate, the diseased state. Although researchers occasionally discover new therapeutic agents after major breakthroughs have elucidated the molecular basis of a disease, more often they must rely on observing for antibiosis or modifying the chemical structures of functionally related chemicals.

With respect to antibiotic agents, some are quite effective at the outset, but over time many organisms become resistant or totally immune to their action. Additionally, very few effective anti-viral agents have ever been developed, and without explicit, detailed knowledge of an infecting organism's physiology, the development of new operative agents remains haphazard.

Thus, there exists a definite need for a methodology enabling the systematic design of new antibiotics and other therapeutic agents that is versatile and inexpensive, yet produces agents that are both extremely specific and effective. The present invention fulfills these needs.

SUMMARY OF THE INVENTION

The present invention provides a methodology of identifying and constructing therapeutic and other agents for use in living organisms that substantially reduces the uncertainty surrounding the development of new antagonists, significantly increasing the scope of materia medica. Moreover, the agent construction of the present invention lends itself readily to simple manufacture, even in large quantities, is extremely effective in use, and attains its improved results without undue cross-reactions.

In a presently preferred embodiment of the invention, by way of example and not necessarily by way of limitation, a stabilized oligonucleotide, preferably in a phosphotriester form, is provided having a base sequence substantially complementary to a portion of messenger ribonucleic acid coding for a biological component of an organism. Due to the complementary nature of the oligonucleotide and the messenger ribonucleic acid, the two components can readily hybridize under appropriate conditions to control synthesis of the organism's biological component and, if the protein is vital to the organism's viability, to act as an antibiotic.

A method, in accordance with the present invention, of developing therapeutic agents may typically include the steps of: providing a base sequence of an organism's nucleic acid that contains at least a portion of the genetic information for a biological component of the organism, and synthesizing an oligonucleotide the sequence of which is derived from the base sequence, for

subsequent hybridization with the messenger ribonucleic acid specific for the biological component. The biological component may be a vital protein, or simply a hormone such as the gonadotropin, follicle stimulating hormone. The order of the base sequence may be determined from deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), preferably messenger ribonucleic acid (mRNA). Alternatively, the desired oligonucleotide base sequence may be determined from the biological component's sequence, as when the biological component is a protein. The preferred oligonucleotide has a minimum of about fourteen or more bases, such as about twenty-three bases, and for increased stability, may be transformed to a more stable form, such as a phosphotriester form, to inhibit degradation during use.

To produce large quantities of the oligonucleotide, it may be synthesized chemically, such as in automated machines, or inserted into a plasmid, such as pBR322, for cloning. The plasmid insertion may be accomplished with linker base sequences, such as GATTC-GAATC or CTAAGCTTAG, which are susceptible to degradation by Hind III restrictive nuclease or Alu I restriction nuclease. When the order of the base sequence has not been determined, the base sequence can be cloned and then cross-hybridized against messenger ribonucleic acid from the other sources to remove base sequences non-specific to the target.

Another aspect of the present invention is a method of selectively controlling activity of one or more biological components in a cell without substantially interfering with the activity of other biological components in the cell. The method includes the steps of

forming an oligonucleotide having a base sequence substantially complementary to a portion of mRNA coding for the specific biological component, and introducing the oligonucleotide into the cell for hybridization with the selected mRNA. This causes blocking of the translation of the mRNA into protein. The oligonucleotide may have at least about fourteen bases or more, such as about twenty-three bases. The target mRNA can code for a protein, such as the hormone, follicle stimulating hormone. This hormone has an alpha and beta chain, and the oligonucleotide should be specific for the mRNA coding for the beta chain to avoid cross-reacting with other gonadatropin mRNA. A suitable oligonucleotide base sequence would be ACCACGCGR$_1$CCR$_2$ATGACGATGTG, wherein R$_1$ is G or T and R$_2$ is also G or T.

In accordance with another aspect of the present invention, a method is provided for inhibiting the infection of a host organism by a foreign organism. This method entails isolating a base sequence containing at least a portion of the genetic information coding for a vital protein from the foreign organism's nucleic acid; synthesizing an oligonucleotide, the order of which is derived from the base sequence and substantially complementary to the messenger ribonucleic acid coding for the protein; and treating the foreign organism with an effective amount of the oligonucleotide to hybridize with a portion of the messenger ribonucleic acid and block translation of the protein. The oligonucleotide, which can be a deoxyribonucleotide, can be transformed to a more stable form, such as a phosphotriester form, to inhibit degradation, and the order of the sequence determined prior to its synthesis. Further, to increase the oligonucleotide's specificity, it may be cross-hybri-

dized against mRNA from different organisms, such as the host organism, to remove non-unique oligonucleotide sequences.

It will be appreciated from the foregoing that the present invention satisfies a long existing need for improved methods of developing therapeutic agents for use in living organisms, and represents a significant advance over previously available methods, principally because it is very versatile, and yet provides a very specific agent against a biological component. Other aspects and advantages of the invention will become apparent from the following more detailed description taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a flow diagram showing the central dogma of molecular biology;

FIG. 2 is a flow diagram showing normal translation of messenger ribonucleic acid (mRNA) into T protein, as well as a synthetic oligonucleotide of the present invention blocking translation of the T protein;

FIG.3 is a list of the viral deoxyribonucleic acid (DNA) code specific for SV-40 T protein, and the related mRNA and oligonucleotide;

FIG.4 is a flow diagram showing the construction of high yield plasmids containing the T protein oligonucleotide;

FIG.5 is a flow diagram showing the use of restriction nucleases to cleave the plasmids to give a purified T protein oligonucleotide;

FIG. 6 is a flow diagram showing the treatment of a DNA sequence to form DNA polyphosphotriesters; and

FIG. 7 is a chart showing the partial amino acid sequence of follicle stimulating hormone, as well as the predicted mRNA sequence and related oligonucleotide family.

DETAILED DESCRIPTION OF THE INVENTION

Referring now to the drawings, and particularly to FIGS. 1 and 2, there is shown the so-called "central dogma" of the molecular biology of life. Basically, it is now accepted that deoxyribonucleic acid (DNA) carries the genetic code for almost all living organisms. The code exists in the form of an organized sequence of four nucleotide bases attached to a phosphorylated, deoxyribose sugar backbone. Generally, DNA exists in the form of a double strand helix formed of two oppositely directed strands, which are held together in an opposing manner by various weak forces.

A primary constituent of these weak forces are the so-called hydrogen bonds that exist between nucleotides on the opposing strands. The four bases, adenine (A), cytosine (C), guanine (G), and thymine (T), form hydrogen bonds generally in only one fashion: A with T and C with G. Thus, by knowing the sequence of one strand, the sequence of the second strand can be readily determined.

Another aspect of the central dogma is that proteins are produced indirectly from the DNA strand, through messenger ribonucleic acid (mRNA). Apparently, mRNA, which has the same structure as single stranded DNA except with a ribose backbone and with uracil (U) replacing thymine, is transcribed directly from one DNA strand and has an essentially opposite base sequence, i.e., if a DNA strand sequence is 5'...ACGT...3' the transcribed mRNA sequence is 3'...UGCA...5'.

An additional aspect of the central dogma relates to the translation of mRNA into proteins.

Briefly, excluding initiation sites and the like, each three nucleotide base grouping (triplet code) codes for one amino acid of a protein. Therefore, by knowing the mRNA sequence of a protein, its amino acid sequence can generally be determined. However, the reverse is not true, that is, knowing the amino acid sequence does not guarantee precise knowledge of the mRNA sequence. This stems from the fact that there exist 64 (4^3) possible triplet codes, yet there exists only about twenty amino acids, allowing some amino acids to have multiple triplet codes.

The similarity in the structure of DNA and mRNA strands creates interesting effects. Most notably, if complementary DNA and RNA strands exist contemporaneously in a solution, under certain established conditions the strands can anneal, forming hybrids. One important factor in proper annealing is the melting temperature, which can be calculated according to Britten et al, Methods of Enzymology 29:363 (1974).

In accordance with the present invention, a synthetic oligonucleotide having a base sequence capable of substantially matching that of a chosen mRNA is provided for hydridization with that mRNA. Once such a hybrid exists, the translation of the mRNA into protein becomes significantly inhibited. If the inhibited protein is vital to an organism's survival, the organism's viability, i.e., either growth or continued life, is jeopardized. Importantly, the oligonucleotide can be designed specifically for the mRNA coding for just one protein, and should not cross-react with mRNA for other proteins.

The method of developing the oligonucleotide entails basically two steps. As described more fully below, a possible first step is to determine the appropriate sequence of the mRNA specific for the protein to be inhibited, and a second step is to manufacture an oligonucleotide complementary to the mRNA. Once made, the oligonucleotide can be treated into a phosphotriester form for increased stability.

A variety of techniques exists for determining nucleic acid base sequences. In many instances the sequence of the mRNA or the gene have been determined and published in the biochemical literature. In fact, researchers have determined the complete nucleotide sequence for the SV-40 virus (Reddy et al, Science 200:494 (1978)). As is well known, an alternative method entails isolating and purifying mRNA in sufficient quantities to permit sequencing studies, but this can prove difficult due to the relative instability and, in some cases, extreme rarity of many mRNA sequences.

Still another method for determining nucleic acid base sequences requires resolving the amino acid sequence from the target protein. After determining the amino acid sequence of the target protein in purified form, a sequential degradation utilizing commercially available protein sequences (e.g. from Beckman Instruments, Fullerton, California) can be used to provide the amino acid sequence. Once this has been obtained, knowledge of the triplet code can be applied to give prospective base sequences. An example of such a process for the hormone glucagon can be found in Tullis et al, Biochemical and Biophysical Research Communications 93:941 (1980).

Once the sequence of the appropriate nucleic acid and the desired mRNA sequence have been determined, an oligonucleotide, such as deoxyribonucleotide, complementary to the mRNA can be constructed. A number of synthetic techniques are known, most typical is the diester approach described by Agarwal et al, Nature 227: 27 (1970), and oligonucleotide synthesizers may be purchased commercially from Vega Biochemicals, P.O. Box 11648, Tucson, Arizona and Biologicals, Inc., Toronto, Canada.

If the desired oligonucleotide sequence is unknown, a suitable oligonucleotide can be prepared as follows. After isolating mRNA from a target organism, multiple copies are made, preferably in the form of DNA, so-called copy DNA (cDNA). This cDNA is then cross-hybridized against mRNA isolated from other organisms, and any cDNA hybridizing is removed. The remaining cDNA is specific only to the target organism, and can serve as the therapeutic agent.

In order to obtain a high degree of specificity, an oligomer of about fourteen or more residues can be constructed. Although shorter sequences will work, longer sequences provide higher specificity. This can readily be seen mathematically. Whereas a ten unit polymer chosen from four bases can have 4^{10} (1,048,576) random combinations, a 20-unit polymer has 4^{20} random combinations, which equals 1.09×10^{12} (1,090,000,000,000).

In spite of the added difficulty in making oligonucleotides of twenty units in comparison to ten bases, it is warranted because the exponential increase

in complexity reduces undesirable cross-reactivity. It has been estimated that a mammalian cell contains about $2x10^8$ nucleotides of RNA complexity or, in other words, approximately 200 million nucleotides of unique sequence mRNA, which is equivalent to about 30,000 mRNA sequences. The probability that one of those sequences contains a randomly chosen 20-unit polymer is approximately one in fifty-five hundred. In comparison, a ten-unit polymer has about a one hundred and ninety to one chance for random cross-reaction.

The present invention is illustrated by, but not limited to, the following examples.

EXAMPLE I

SV-40 virus manufactures a vital protein commonly known as the "T protein" or "T antigen protein". As noted earlier, the complete genetic code for the SV-40 virus has been determined, and it is known that residues 5091 to 5071 on the viral genome code for a portion of the T protein mRNA. The sequence of these residues, the viral T protein mRNA sequence, and the designed T protein specific oligonucleotide are shown in Fig. 3. In this case, the T protein specific oligonucleotide is complementary to the viral T protein mRNA, and identical to the portion of the viral DNA code.

Prior to testing the effectiveness of the T protein specific oligonucleotide in vivo, the oligonucleotide can be mixed with total mRNA from an organism to check for cross-reactivity. If it hybridizes, then a different portion of the viral genome coding for the T protein should be utilized. Otherwise, the oligonucleotide is ready for further testing.

Further testing requires growth of S-40 virus. For purposes of these experiments, SV-40 virus is grown and titered in African green monkey cells, such as the cell line BSC-1, according to Hopps et al, Journal of Immunology 91:416 (1963). The identity of the virus can be confirmed by the following methods:

a) checking for tumor production after innoculation of the newborn hamsters with the virus;

b) neutralization of the virus by anti-SV-40 antiserum; and

c) reaction of the infected cells with anti-SV-40 T antigen directed antibodies prepared by standard techniques.

The isolation of SV-40 mRNA can be accomplished as follows. Total RNA is first obtained by the guanidine hydrochloric acid extraction procedure using glassware previously baked and treated with diethylpyrocarbonate to remove traces of RNase as taught by Cox et al, Methods in Enzymology 12B:120 (1968). The A+ RNA is isolated on oligo-dTcellulose, which can be obtained from Collaborative Research, Waltham, Massachusetts, or P. L. Biochemicals, Inc., Milwaukee, Wisconsin, utilizing the technique described by Bantle et al, Analytical Biochemistry 72:413 (1976). The RNA fractions are assayed for purity and intactness by electropheresis according to the method described in Bailey et al, Analytical Biochemistry 70:75 (1976). Also, the RNA can be assayed for translatability in the wheat embryo *in vitro* system described in Marcus, et al, Methods in Enzymology: 30:749 (1974). The *in vitro* translation products are monitored on sodium lauryl sulphate 9% polyacrylamide gels as described in Laemmeli, Nature 227:680 (1970).

This purified A+ mRNA containing the viral mRNA sequences can hybridize to the synthetic oligonucleotide at 37°C in 0.5 M sodium phosphate buffer, pH 6.8, containing 0.2% sodium lauryl sulphate. Solutions containing about 1 mg A+ mRNA and synthetic oligonucleotide at a concentration of about 100 ug/ml are heated to 100°C for I-2 minutes, then cooled to 37°C and allowed to anneal. The extent of the hybridization reaction as a function of time may be monitored on a gel filtration column.

Actually, while any theoretically suitable temperature may be used for the hybrid formation, temperatures ranging from 0°C to about 80°C provide for good hybridization, but preferred temperatures range from about 10°C to about 40°C. Generally, the optimal annealing temperature for the formation of specific hybrids is believed to be about 20°C to 25°C below their melting temperature. Synthetic oligonucleotides operating at 37.5°C should thus be designed on the basis of their base sequence and length, such that the melting temperature is between about 57°C and 62°C when tested under approximately physiological conditions.

For hybridization testing the ratio of the synthetic oligonucleotide to its mRNA complement is generally about 30:1. Lower ratios are acceptable, however, sequences below about 3:1 can cause lower hybrid formation. Control reactions utilizing yeast RNA or globin mRNA can be used, and should show no detectable hybrids, indicating hybridization specificity only to SV-40 mRNA. Also, thermal denaturation profile studies and comparison of the kinetics of hybridization can confirm that the synthetic oligonucleotide reacts only

with SV-40 mRNA sequences.

Once it is shown that the oligonucleotide hybridizes to the isolated SV-40 mRNA, in vitro translation tests can be attempted utilizing the wheat embryo system (described previously) to show that the hybrid is not translated. Basically, upon introduction of SV-40 mRNA into the wheat embryo system, the system produces large T antigen protein. However, when an equal amount or more of synthetic oligonucleotide is also added to the system, T antigen protein synthesis can be substantially inhibited, without interference with synthesis of other SV-40 proteins whose mRNA was also introduced.

Testing of the oligonucleotide in vivo can be accomplished by adding the oligonucleotide to cultures of cells infected with SV-40. Synthesis of T antigen protein should be inhibited signficantly in about six hours, and SV-40 growth should be strongly inhibited within about 24 hours. The growth of control cultures should be largely unaffected.

The synthetic oligonucleotide of the present invention may be mass produced according to common cloning techniques, such as those developed in the art to clone the gene for proinsulin. Alternatively, the oligonucleotide can be chemically synthesized in commercially available equipment (described previously). Briefly, the cloning method entails enzymatic insertion of the oligonucleotide into a bacterial gene carried on a larger piece of DNA, known as a plasmid. The plasmid can be incorporated into a suitable host bacteria, and multiple copies made as the bacteria multiply as in Boyer and Cohen, U. S. Patent No. 4,237,224.

More particularly, and with reference to FIGS. 4 and 5, the cloning plasmid designated as pBR322, available from Bethesda Research Labs, Inc., Rockville, Maryland, can be used to mass produce the T protein specific oligonucleotide. Using standard techniques, the oligonucleotide is converted to double stranded form and then a terminal 5′PO_4 is added to each of the 5′ termini with polynucleotide kinase to permit subsequent joining through T-4 ligase. The reaction conditions for forming the 5′ termini can be found in Richardson, Progress in Nucleic Acids Research 2:815 (1972).

After purification of the double stranded oligonucleotide by chromatography on hydroxylapatite columns, it is inserted into the plasmid. Because the oligonucleotide is blunt ended, the plasmid should not have uneven or "sticky" ends. To remove sticky ends from the plasmid, S1 nuclease or other single strand specific nucleases can be utilized. A general description of methods for using restriction nucleases can be found in Nathans and Smith, Annual Review of Biochemistry 44: 273 (1975).

For best results, a linker system between the oligonucleotide and the plasmid can be utilized, specifically a linker having both Hind III and Alu I enzymatic cleaving sites. As seen in FIG. 4, one such linker has a sequence: 5′... CTAAGCTTAG...3′. This sequence represents a double stranded, bisymmetric molecule containing a recognition sequence both for Alu I (AGCT) and for Hind III (AAGCTT). Utilizing DNA ligase under standard conditions, this molecule can be ligated to the oligonucleotide to form linker-oligonucleotide-linker molecules. Similarly, the linked oligonucleotide can be

introduced into linearized blunt-ended, Hind III cleaved pBR322 carrier molecules.

After ligation, the plasmid has resumed its covalently closed circular configuration with the linker-oligonucleotide incorporated, all of which is shown in FIG. 5 as pT-protein oligonucleotide. The recircularized plasmid is then used to transform a suitable bacterial host such as E. coli. The methods for transformation and selection of transformants are known in the art and described in detail in Cohen and Boyer, U.S. Patent No. 4,237,224.

Once the transformed bacteria containing the ligated plasmid p-oligonucleotide have been grown to high density and produced large amounts of the ligated plasmid, the oligonucleotide is ready for purification. After the plasmid has been removed from the mature cells, the plasmid is treated with appropriate restriction endonucleases. As illustrated in FIG. 5, the plasmid is first cleaved with Hind III to give various by-products, including linker-T-protein-oligonucleotide-linker and fragments of the original plasmid. These are readily separated utilizing gel electrophoresis or high pressure liquid chromotography. Further cleavage of the isolated linker-oligonucleotide-linker with the endonuclease Alu I yields pure double stranded oligonucleotide and partially degraded linker. These can also be separated based on their size differences.

As shown in FIG. 6, the oligonucleotide can then be modified to a nuclease resistant phosphotriester form utilizing the reaction described in Miller et al, Biochemistry 16:1988 (1977). Basically, the oligonucleo-

tide is first acylated using 50% acetic anhydride-pyridine during an overnight incubation period. The product is precipitated and isolated from ether. The phosphotriester can then be formed utilizing 30% ethanol in anydrous 2, 6 lutidene (30%), NN-dimethyl formamide (30%) and p-toluene sulfonyl chloride (17%), and reacting for about 6 hours. The protecting acetyl groups are then hydrolyzed by the addition of 0.5 volumes of concentrated ammonium hydroxide, followed by incubation for about 1 hour at 55°C. The final oligonucleotide product in the ethyl phosphotriester form can then be isolated on paper chromotography or high pressure liquid chromotography.

It is believed that transforming the oligonucleotide to a phosphotriester form will improve the oligonucleotide's stability in vivo due to an enhanced resistance against various degradative enzymes. However, the oligonucleotide will eventually degrade because of spontaneous de-ethylation, which leaves the molecule unprotected. Indeed, by controlling the initial level of ethylation, the in vivo degradation rate can be controlled. A further advantage of a phosphotriester form is believed to be an increase in the oligonucleotide's ability to penetrate a cell membrane.

EXAMPLE 2

A synthetic oligonucleotide capable of inhibiting the synthesis of follicle stimulating hormone (FSH), a protein hormone produced by the pituitary that functions in the maturation of ova in females and sperm cells in males, can also be constructed. It is known that FSH is composed of two chains, alpha and beta, the amino acid sequence of which has been determined for several animal

species. Interestingly, the alpha chain of FSH is common to other gonadotropic hormones, including thyroid stimulating hormone, luteinizing hormone, and chorionic gonadotropin, while the beta chain varies. Therefore, to selectively shut off the synthesis of FSH without substantially affecting the other gonadotropins, the oligonucleotide must be specific for the mRNA coding for the beta chain.

The sequence of the beta chain amino acids 32 through 40 is shown in FIG. 7. As discussed earlier, it is possible to predict the mRNA base sequence for these amino acids, although not with absolute certainty. The points of uncertainty are indicated by the letter "X" in the predicted mRNA sequence. Thus, the resultant oligonucleotide family consists of eight possible 26 base sequences; the potential alternate bases are shown in parentheses below the primary base sequence.

By beginning with the projected mRNA sequence for the 33rd through 40th amino acids, it can be seen that four different 23 base oligonucleotides exist that could correspond to the FSH mRNA. The sequences could be as follows, reading from the 5′ end: GTGTAGCAGTAGCCGGC-GCACCA, GTGTAGCAGTATCCGGCGCACCA, GTGTAGCAGTAGCCTGCGCACCA, and GTGTAGCAGTATCCTGCGCACCA.

One of these four sequences should be precisely correct and thus able to hybridize fully with the FSH mRNA. To determine the best sequence, a hybridization test against FSH mRNA, with subsequent purification on hydroxylapatite or other suitable column, can be performed as previously described. Once the best sequence has been determined, it is placed in a plasmid or

chemically synthesized, as described above, for bulk synthesis. This oligonucleotide should substantially inhibit the synthesis of FSH in vivo.

From the foregoing, it will be appreciated that the present invention provides a systematic method of designing new therapeutic agents for use in living organisms and that this method is versatile and inexpensive. Further, the oligonucleotide produced in accordance with the present invention is extremely effective and specific, enabling selective control of protein synthesis in a living organism.

While several particular forms of the invention have been illustrated and described, it will be apparent that various modifications can be made without departing from the spirit and scope of the invention. Accordingly, it is not intended that the invention be limited, except as by the appended claims.

CLAIMS

1. A method of developing therapeutic agents comprising the steps of: providing a base sequence of an organism's nucleic acid, said base sequence containing at least a portion of genetic information for a biological component of said organism; and synthesizing an oligonucleotide, the sequence of which is derived from said base sequence, for hybridization with messenger ribonucleic acid specific for said biological component.

2. The method of Claim 1 further including the step of: transforming said oligonucleotide into a more stable form to inhibit degradation by said organism.

3. The method of Claim 2 wherein said more stable form is a phosphotriester form.

4. The method of Claim 1 wherein said biological component is a protein.

5. The method of Claim 4 wherein said oligonucleotide is a deoxyribonucleotide.

6. The method of Claim 1 wherein the base sequence comprises about fourteen or more bases.

7. The method of Claim 1 wherein the base sequence comprises about twenty-three bases.

8. The method of Claim 1 wherein the order of said base sequence is determined from ribonucleic acid or deoxyribonucleic acid specific for said biological component prior to synthesizing the oligonucleotide.

9. The method of Claim 1 further comprising the step of: inserting said oligonucleotide into a plasmid for cloning.

10. The method of Claim 9 wherein said oligonucleotide is inserted into said plasmid with a linker base sequence.

11. The method of Claim 10 wherein said linker sequence is GATTCGAATC or CTAAGCTTAG.

12. The method of Claim 10 wherein the linker is susceptible to partial degradation by Hind III or alu I restriction nuclease.

13. The method of Claim 1 further comprising the

step of: cross-hybridizing the base sequence against nucleic acid from at least one source other than said organism, whereby the base sequence is more specific to said organism.

14. A method of selectively controlling activity of one or more specific biological components in a cell without substantially interfering with the activity of other biological components of said cell, said method comprising the steps of: forming an oligonucleotide having a base sequence substantially complementary to a portion of messenger ribonucleic acid coding for said biological component; and introducing said oligonucleotide into said cell.

15. A method of inhibiting the infection of a host organism by a foreign organism, said method comprising the steps of: isolating a base sequence from said foreign organism's nucleic acid, the base sequence containing at least a portion of genetic information coding for a protein vital to said foreign organism's viability; synthesizing an oligonucleotide, the order of which is derived from said base sequence to be substantially complementary to messenger ribonucleic acid coding for the protein; and treating said foreign organism with an effective amount of oligonucleotide to hybridize with a portion of said messenger ribonucleic acid and block translation of said protein, thereby inhibiting the viability of the foreign organism.

16. An agent for use in controlling synthesis of a protein, said agent comprising an oligonucleotide in a stabilized form to inhibit degradation and having a nucleotide sequence substantially complementary to a base sequence of at least a portion of messenger ribonucleic acid coding for said protein.

FIG. 1

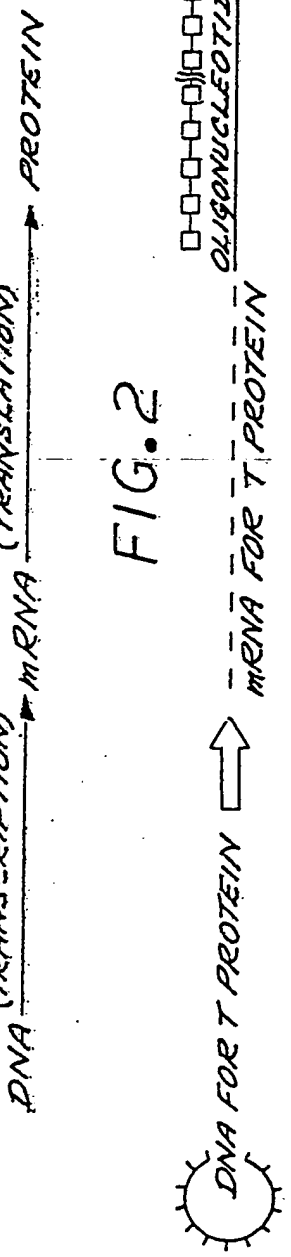


FIG. 2

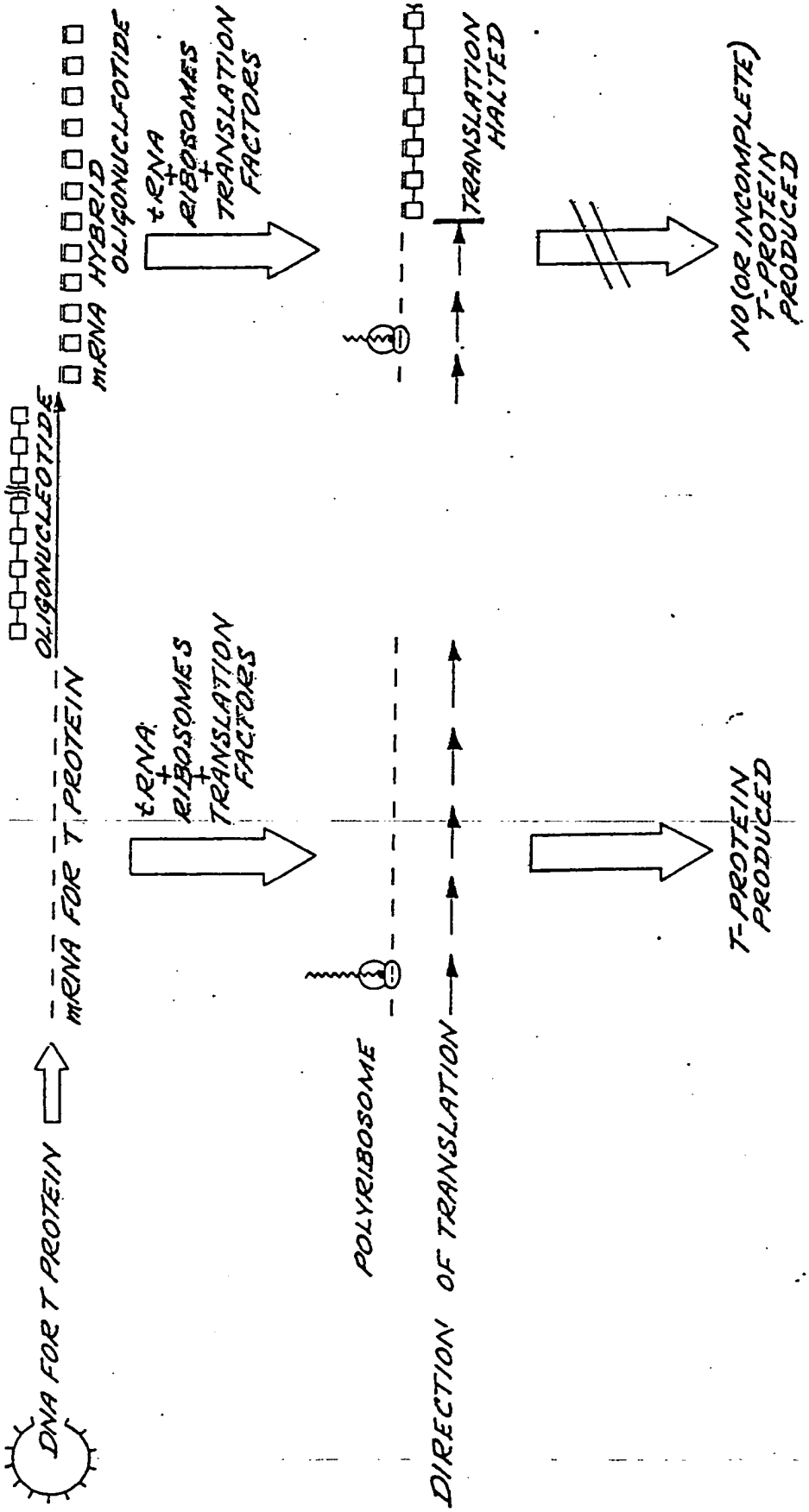


FIG. 3

OLIGONUCLEOTIDE SPECIFIC FOR SV-40 T PROTEIN

VIRAL DNA (CODING) 3'...GAAACGTTTCTACCTATTTC...5'

VIRAL T PROTEIN mRNA 5'...CUUUGCAAAGAUGGAUAAAG...3'

T PROTEIN SPECIFIC OLIGONUCLEOTIDE 3'...GAAACGTTTCTACCTATTTC...5'

FIG. 7

OLIGONUCLEOTIDE SPECIFIC FOR FOLLICLE STIMULATING HORMONE

PROTEIN SEQUENCE:
N TERMINUS....THR TRP CYS ALA GLY TYR CYS TYR THR...C TERMINUS

mRNA PREDICTED:
5' END ACX UGG UGC GCX GGX UAC UGU UAC ACX 3' END

FSH OLIGONUCLEOTIDE FAMILY:
3' END TCG(T) ACC ACC CGG(T) CCG(T) ATG ACG ATG TG- 5' END

FIG. 4

CONSTRUCTION OF HIGH YIELD PLASMID CONTAINING THE T PROTEIN OLIGONUCLEOTIDE

FIG. 5

PRODUCTION OF SV-40 T PROTEIN OLIGONUCLEOTIDE FROM pT OLIGONUCLEOTIDE USING RESTRICTION NUCLEASE CLEAVAGE TO RELEASE FRAGMENTS.

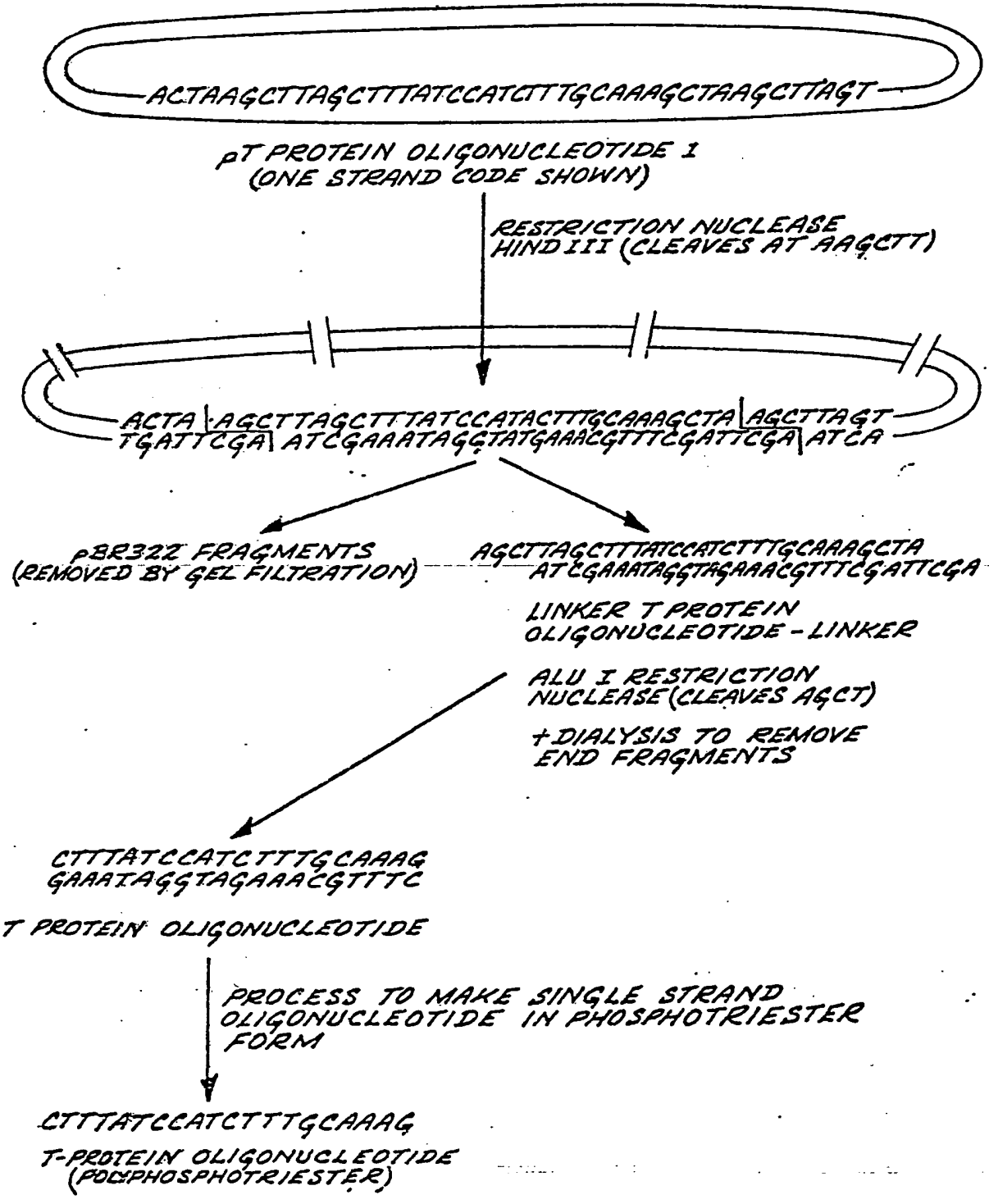
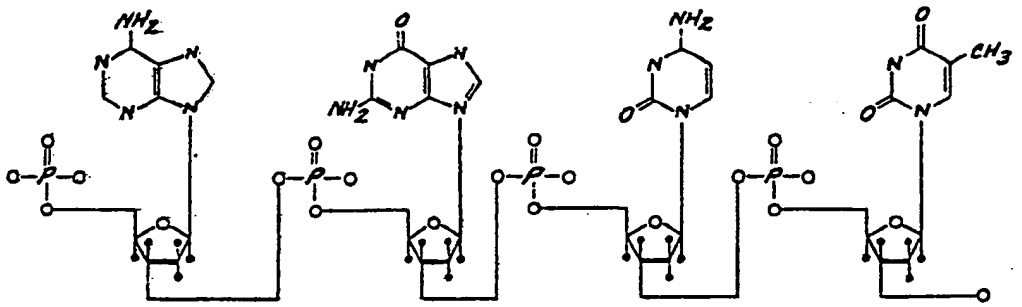
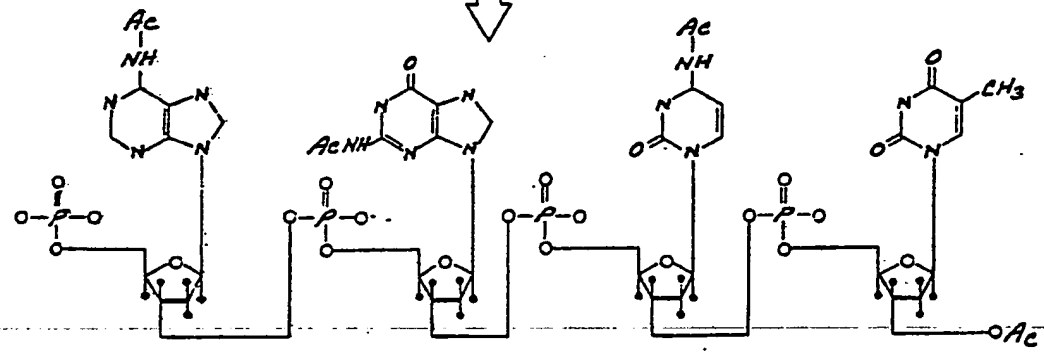


FIG 6



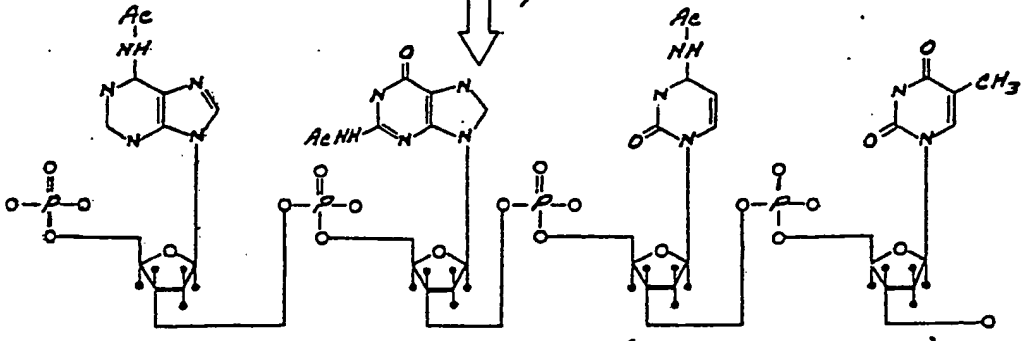
DNA SEGMENT

ACETIC ANHYDRIDE



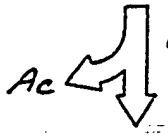
ACETYLATED DNA (BASE PROTECTED)

30% ETHANOL
NN-DIMETHYLFORMAMIDE
17% TOLUENE SULFONYL CHLORIDE
2,6 LUTIDENE



DNA POLYPHOSPHOTRIESTER (BASE PROTECTED)

OH^-



DNA POLYPHOSPHOTRIESTER

International Application No PCT/US82/01447

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC:3 C07H 21/02, C07H 21/04, C12N 15/00, C12Q 1/68, C12P 21/00, C12P 19/34

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
U.S.	424/200; 435/6,68,70,91,172,317; 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

MEDLINE 1966-82
BIOSIS PREVIEWS 1969-83
LIFE SCIENCES COLLECTION 1978-82

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	US, A, 4,237,224, PUBLISHED 02 DECEMBER 1980, COHEN ET AL.	1-16
A,P	US, A, 4,321,365, PUBLISHED 23 MARCH 1982, WU ET AL.	1-12,16
A	GB, A, 2,068,971A, PUBLISHED 19 AUGUST 1981, CAREY ET AL.	1-16
A	N, WALLACE ET AL, NUCLEIC ACIDS RESEARCH, VOLUME 6, NUMBER 11, 1979, PAGES 3543-57.	1-16
X	N, INGLIS ET AL, VIROLOGY, VOLUME 78, 1977, PAGES 522-36.	1,4,6,7,14-16
A	N, HASTIE ET AL, PROC. NATL. ACAD. SCI., USA, VOLUME 75, NUMBER 3, MARCH 1978, PAGES 1217-21.	1-16
A	N, ALVARADO-URBINA ET AL, SCIENCE, VOLUME 214, 16 OCTOBER 1981, PAGES 270-74.	1-12,16
X	N, PATERSON ET AL, PROC. NATL. ACAD. SCI., USA, VOLUME 74, NUMBER 10, OCTOBER 1977, PAGES 4370-74.	1,4,5-7,9,14, 16
X	N, ITAKURA ET AL, SCIENCE, VOLUME 209, 19 SEPTEMBER 1980, PAGES 1401-1405.	1-12,16

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29 DECEMBER 1982	06 JAN 1983
International Searching Authority	Signature of Authorized Officer
ISA/US	JAMES MARTINELL

Form PCT/ISA/210 (second sheet) (October 1981)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	N, PATERSON ET AL, PROC. NATL. ACAD. SCI., USA, VOLUME 74 NUMBER 10, OCTOBER 1977, PAGES 4370-74.	2,3,8,10-13 15
Y	N, INGLIS ET AL, VIROLOGY, VOLUME 78, 1977, PAGES 522-26.	2,3,5,8-13

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.